Mobile-Phase Cleanup Using Solid-Phase Extraction Disks

Troubleshooting background peaks in a chromatographic run can be a time-consuming and challenging proposition. For gradient high performance liquid chromatography (HPLC) methods, minute contamination of the mobile phase during normal preparation can compromise the baseline quality and chromatographic data significantly. The authors describe a fast, simple procedure for purifying mobile phase that uses solid-phase extraction disks during the filtration step for mobile-phase preparation. The procedure removed more than 95% of contaminants, so it is effective and suitable for various gradient HPLC methods.

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A common problem with high performance liquid chromatography (HPLC) is the appearance of spurious peaks, humps, and dips in the baseline. A survey of the liquid chromatography (LC) troubleshooting literature from *LCGC* alone reveals more than 20 separate discussions of this problem (1). Although baseline interferences occur in every type of chromatography, background peaks and features are perhaps most pernicious for gradient elution HPLC methods (2–4). In contrast with isocratic HPLC in which the column and mobile phase are at equilibrium, the mobile-phase properties vary, often dramatically, in gradient HPLC during the run. Before each gradient HPLC injection, a column must be equilibrated to the initial conditions with a weak mobile phase. Typically this requires the flushing of 5–10 column volumes of the weak mobile phase (mobile phase A) through the column. Hydrophobic contaminants in the weak mobile phase will adsorb in the head of the column and be eluted as peaks in the subsequent gradient run. Because the equilibration volume is typically at least two orders of magnitude greater than the volume of the injected sample, it is not surprising that trace-level contaminants can cause significant interferences. The resultant background peaks and features diminish the accuracy, precision, and sensitivity of a method and compromise peak identification. At worst, the chromatographic data are rendered unusable by the noisy baseline and anomalous peaks. Gradient background problems are so common and difficult to troubleshoot that many analysts are reluctant to implement gradient methods in high-throughput, good laboratory practice–regulated, and good manufacturing practice–regulated laboratories.

In contrast with isocratic HPLC, using high-purity solvents and reagents in gradient HPLC does not always ensure a clean background. Minute degradants or contaminants in water, acetonitrile, and trifluoroacetic acid have been shown to contribute gradient background peaks (2,3,5–8). Even clean, fresh mobile phase easily can become unsuitable during typical mobile-phase preparation procedures. The use of plastic or rubber laboratory supplies — such as containers, stoppers, Parafilm, and gloves — can introduce phthalates and other UV-absorbing species into the solutions they contact (5,9). Background peaks also can originate from less-obvious sources such as helium spargers (3,5), dishwashers, and pH electrodes (4). Because small amounts of mobile-phase contamination are concentrated by the gradient, the most conscientious analysts using the purest reagents often cannot meet the

purity demands of HPLC gradient methods, especially when performing detection at low wavelengths.

Because the potential for contamination varies from analyst to analyst and day to day, isolating and removing the contamination source from the laboratory can be a long and frustrating process that is not well suited to high-throughput laboratories. Although the problem of background peaks is quite common, a search of the literature failed to reveal a large number of solutions for this problem. Many analysts insert a cleanup column directly on the HPLC instrument upstream from the solvent mixer (3,10,11). Although this on-line cleanup technique is fast and effective, it requires frequent maintenance and dedicated instrumentation, and it is not suited to low-pressure mixing systems. In addition, the cleanup column contributes backpressure; therefore, this technique can be unsuitable for fast-LC methods. The off-line removal of contaminants by passing solutions through activated silica, alumina, or C18 columns (9) or solid-phase extraction (SPE) cartridges (10) also has been effective, but we have found it difficult to adapt to large volumes of solution without requiring specialized glassware or apparatus.

We sought a simple procedure for removing common laboratory contaminants from mobile phase prepared in a typical manner. SPE disks can be added to a simple filtration apparatus during the filtration and degassing step of mobile-phase preparation. Although SPE has been used extensively for sample preparation in environmental, food, and pharmaceutical analysis, the use of these disks for mobile-phase preparation appears novel. In this article, we describe a fast, effective use of SPE disks for mobile-phase cleanup of several typical HPLC mobile phases.

Experimental

Mobile-phase preparation: To test the efficacy of the cleanup procedure under worst-case conditions, we prepared 2 L of the aqueous portion of the mobile phase with HPLC-grade water and reagents, and then we deliberately adulterated the solution by adding 5–10 drops of pH-electrode filling solution, a large Latex glove, four squares of Parafilm, a cut-up plastic transfer pipette, and 0.5 mL of concentrated dish-washing fluid for automatic glassware washers. After transferring the adulterated solutions to a plastic carboy, we filtered the contaminated solutions through a GMF glass-fiber filter (Whatman Inc., Clifton, New Jersey) and two types of SPE disks: activated-carbon (SuperClean aqueous mobile-phase purification filters, Alltech Associates, Inc., Deerfield, Illinois) and poly(styrene–divinylbenzene) (PS–DVB) (Empore SDB-XC, 3M, St. Paul, Minnesota).

After assembling a vacuum filtration apparatus, we soaked each disk in acetonitrile for 1–2 min and then washed each with three 100-mL aliquots of acetonitrile and discarded the filtrate after each aliquot. After filtering and discarding a single 50-mL aliquot of solution, we filtered the remaining 200–1000 mL of solution into a clean flask. We prepared the mobile phase by adding the specified fraction of acetonitrile to the extracted solution. The typical vacuum level (ΔP) was approximately 0.8 bar during the filtration step. We were careful to clean all apparatuses extensively between steps to avoid cross-contaminating the mobile-phase batches. In the investigation of trifluoroacetic acid removal by SPE, we prepared the mobile phase in a similar manner but without the addition of contaminants.

Chromatography: We performed all separations using either an Agilent model 1050 or model 1100 liquid chromatograph equipped with a quaternary pump, autoinjector, and UV detector (all from Agilent Technologies, Inc., Wilmington, Delaware). Table I outlines our chromatographic methods, which encompass a range of mobile-phase chemistries. After washing the system with 50:50 (v/v) acetonitrile–water, we equilibrated the column for at least 20 min at |the start conditions for the gradient. After multiple injections of diluent and test mix solution, we washed the system again with 50:50 (v/v) acetonitrile–water to avoid cross-contaminating the next set of analyses. For each chromatographic method, we used the same the sample solutions and mobile phase B in an effort to isolate interferences arising solely from contaminated solution in mobile phase A.

Results and Discussion

Removal of background peaks: We assessed the level of contaminants in untreated mobile phases by filtering the aqueous solutions through a glass-fiber filter that would not be expected to remove contaminants. As shown in the glass-fiber-filtered diluent blank chromatogram in Figure 1, the deliberate contamination of mobile phase A

Table I: Summary of HPLC gradient methods used in investigation

		Gradient*						
Method	Aqueous Component	Mobile Phase A (v/v Aqueous–Acetonitrile)	Mobile Phase B (v/v Aqueous–Acetonitrile)	Time (min)	% B	Column	Flow Rate (mL/min)	UV Detection Wavelength (nm)
Method A	0.044% Trifluoroacetic acid (pH 2.3)	90:10	5:95	0; 3.4; 10	10; 13; 85	100 mm × 4.6 mm, 3.5-µm d_p Waters Symmetry C18	1.5	226
Method B	0.05 M monobasic ammonium phosphate (pH 2.9)	70:30	22:78	0; 60	0; 100	250 × 4.6 mm, 5-µm d_p Inertsil ODS2	1.0	228
Method C	0.05 M monobasic potassium phosphate (pH 7.1)	98:2	78:22	0; 10; 15	0; 100; 100	100 mm × 4.6 mm, 3-µm d_p Hypersil HyPurity Elite C18	1.5	230
Method D	0.020 M ammonium acetate (pH 10.0)	95:5	5:95	0; 2; 32	0; 0; 100	100 mm × 4.6 mm, 3.5-µm d_p Waters Xterra MS C18	2.0	282

*The gradient program could include high-organic solvent flush and reequilibration steps that are not shown.

resulted in significant background peaks in both the early and late portions of the gradient for method D. The total peak area of background peaks in the glass-fiber-filtered control corresponds to nearly 17-fold the area of the main peak in this assay. Considering that this assay specifies quantitation and reporting of all peaks 0.1% or more of the peak area of the parent compound, we would need to track and evaluate nearly 50 system-related peaks in each sample. Moreover, because we would expect these peaks to interfere with the assay, it would be difficult to perform accurate quantitation of the main peak or to detect and identify low-level impurities.

When mobile phase was treated using the SPE disks, both the number and peak area of background peaks were reduced dramatically, as shown in Figure 1. In the case of the mobile phase made with the PS–DVB extracted solution, the blank injection was clean, with 99% of the observed background peaks removed by the SPE disk.

Because the extraction efficiency of SPE disks would be expected to be dependent upon the solution type, pH, and ionic strength, we investigated the efficacy of the cleanup procedure on a range of solutions typically used in HPLC (Table I), including acidic (methods A and B), neutral (method C), and basic (method D) solutions. Figure 2 compares the percentage of contaminants removed by the filtration procedure for all four methods. For the first blank injection, the background peak areas were decreased significantly by the use of activated-carbon or PS–DVB extraction filtration. The total background peak levels in these blanks still were much higher than acceptable (more than 10% of the area of the main peak in the assay). However, the analysis of the first injection of the blank represents a worst-case condition — as expected, the background peak levels decreased significantly on subsequent injections. Although both SPE disks improved the blank background levels, the PS–DVB extraction disk removed 96–99% of the total peak area in the first blank for all four methods investigated.

One possible explanation for the enhanced efficiency of the PS–DVB SPE disks in removing contaminant peaks could arise from the fact that peak detection for methods A–D occurred at wavelengths (~230 nm and 280 nm) near the $\pi \rightarrow \pi^*$ electronic transition for many aromatic molecules. Because π–π interactions are predicted to be a key retention mechanism for PS–DVB SPE extraction disks, the PS–DVB mobile-phase cleanup is quite selective for the same compounds that would be detected at the selected wavelengths, which resulted in clean baselines when using this cleanup procedure.

Although the mobile-phase extraction efficiencies of activated carbon and PS–DVB were comparable for the acidic solutions used in methods A and B, the efficacy of the carbon SPE disk appeared to decrease as the solution pH increased (Figure 2). For the pH 10 ammonium acetate buffer used in method D, only 50% of the contaminants in the first injection were removed by the carbon SPE disk compared with 99% by the PS–DVB SPE disk. In contrast with the π–π interactions exhibited by the PS–DVB SPE disk, the activated-carbon SPE disk retains compounds mainly on the basis of van der Waals interactions, which are pH dependent under some circumstances. Additional studies would be necessary to assess whether the effect is truly pH related or arises from other variables, such as solution type, that we used in the study.

Effect on system suitability: Clearly, the SPE cleanup is effective for removing common mobile-phase contaminants. However, it also is important to ensure that the procedure does not remove solution additives or alter the pH of the mobile phase, which potentially could change the retention and resolution. To assess the effect of mobile-phase cleanup on system suitability, we made a series of test mix injections following blank injections using method A. As shown in Figure 3, the PS–DVB- and activated carbon–extracted mobile phases exhibited elution profiles that were nearly identical to the glass-fiber filtered mobile phases. Although the detection of low-level impurities — particularly late eluters — was improved dramatically by the mobile-phase cleanup procedure, the retention times and resolution essentially were unchanged when compared with a glass-fiber-filtered control. The results for methods B, C, and D were quite similar, with only minor changes in analyte retention times (no more than 2.5% in all cases) for extracted mobile phases compared with the glass-fiber-filtered control. It is important to note that the contaminants themselves, particularly soap residue, also could modify the separation. Removing the contaminants therefore could have resulted in differences in separation between the filtered and extracted mobile phases. For these methods under these conditions, however, this does not appear to be the case. These results suggest that SPE is selective for typical mobile-phase contaminants and can produce similar mobile phase to a glass-fiber-filtered control.

Although the results shown in Figure 3 are promising in terms of their ability to predict system suitability, it is important to note that the experiment was not designed to address changes in solution composition introduced by the vacuum extraction process itself. We were concerned that the potential for solution changes during vacuum filtration, which was not assessed in previous experiments, was particularly great for method A, which contains trifluoroacetic acid, a volatile component in the aqueous phase. Accordingly, we decided to investigate whether the vacuum filtration process caused losses in trifluoroacetic acid

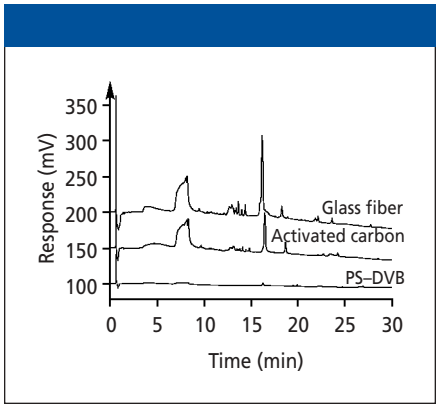

Figure 1: Comparison of diluent blank injection chromatograms for method D using various aqueous-phase pretreatments. All chromatograms are first injections from the same vial after a 20-min equilibration at the start conditions for the gradient. For clarity, chromatograms are shown displaced 50 mV from each other with a 100 mV signal at the start of the chromatogram.

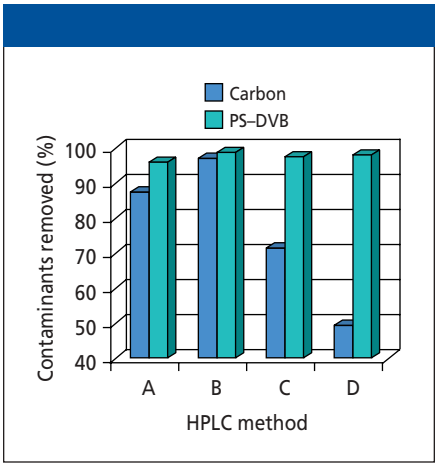

Figure 2: Comparison of mobile-phase contaminant removal assessed in the first injection of a blank for four gradient methods using various aqueous-phase cleanup procedures.

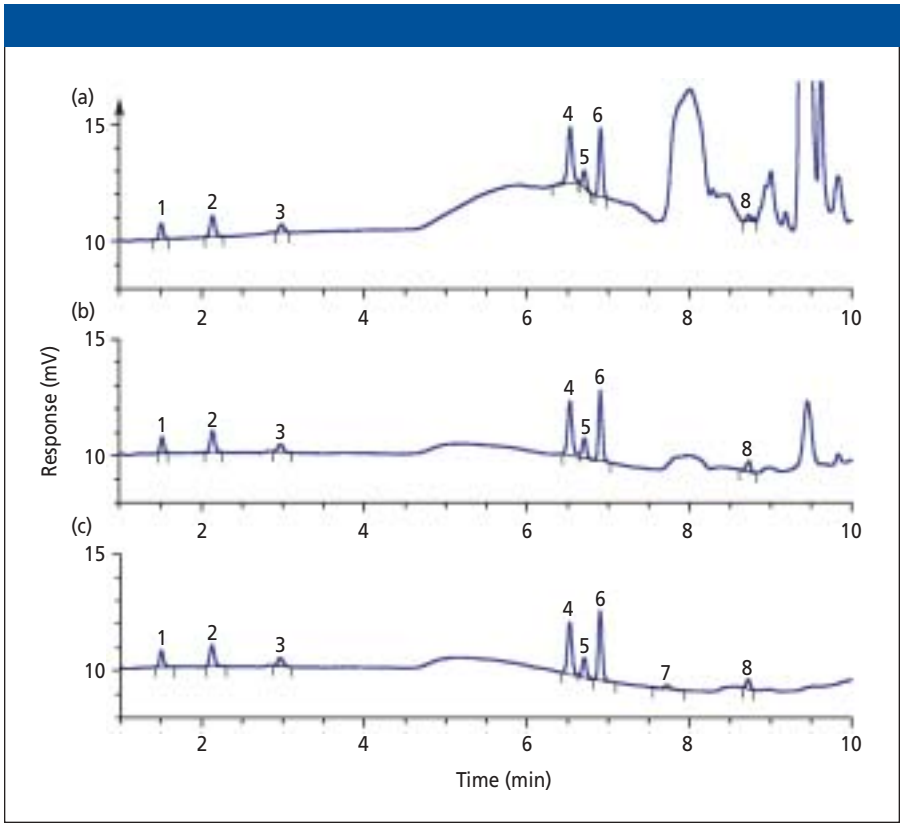

Figure 3: Comparison of test-mix chromatograms for method A using (a) glass-fiber, (b) activated-carbon, and (c) PS–DVB aqueous-phase pretreatments. Peaks 1–8 are impurities spiked at 0.3 to 1.9% area of the active ingredient peak, which was not included in the test mix.

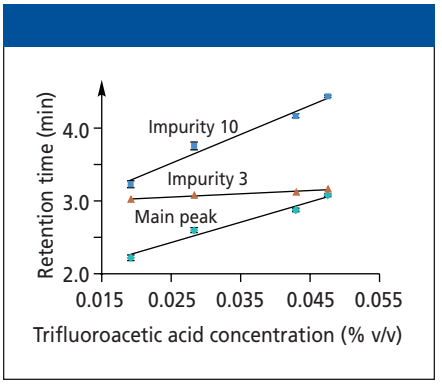

Figure 4: Retention of the main peak and impurities separated using method A as a function of the trifluoroacetic acid concentration of mobile-phase A when prepared in a typical manner. Error bars represent the 95% confidence interval for four replicate injections and are within the dimensions of the marker when not visible.

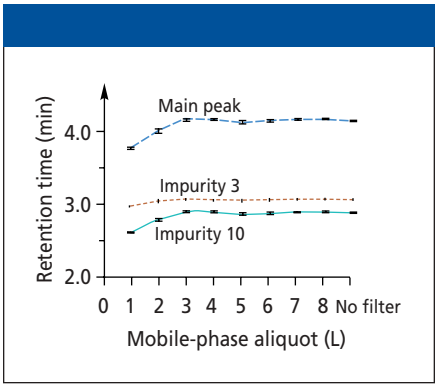

Figure 5: Retention of the main peak and impurities in method A eluted with mobile phase made from successive aliquots of unadulterated trifluoroacetic acid solution extracted with a single PS–DVB SPE disk. Error bars represent the 95% confidence interval for four replicate injections of each extracted fraction and eight replicate injections for the unfiltered fraction.

solution composition in typical, unadulterated solutions.

The data in Figure 4 show the decreased retention of the main peak and impurity 10 when less trifluoroacetic acid was used to prepare the mobile phase without a filtration step. These compounds were not included in the test mix shown in Figure 3 and therefore were chromatographed in a separate series of experiments to probe the chromatographic effect of filtration with the styrene–divinylbenzene SPE disk.

Investigation of trifluoroacetic acid removal by SPE: Trifluoroacetic acid can be lost during SPE as a result of evaporation during vacuum filtration or adsorption by the extraction media. These causes can be studied and addressed independently. Evaporation, for example, can be overcome by using pressure filtration rather than vacuum filtration. Adsorption can be overcome by pre-equilibration of the extraction media.

To investigate whether either of these possible causes was affecting the chromatography for method A, we prepared 10 L of 0.044% trifluoroacetic acid in water (v/v) and extracted the solution in 1-L aliquots using the same extraction disk. Next, we used the trifluoroacetic acid solution aliquots to prepare mobile phase A for method A, and we used an unextracted portion of the solution to prepare an unfiltered control mobile phase. If the SPE process has no effect on the trifluoroacetic acid levels, then the extracted and unextracted mobile phases would exhibit identical elution profiles for these solutes. If trifluoroacetic acid is lost due to evaporation, then all mobile-phase preparations would be expected to behave differently than the unfiltered control. If trifluoroacetic acid is lost due to adsorption, then initial aliquots would differ but no further change in retention would be expected after the disk was equilibrated adequately.

When the main peak and impurities were chromatographed using method A, the mobile phase made with the first 2 L of extracted trifluoroacetic acid solution using a single PS–DVB SPE disk exhibited lower retention times than both the unfiltered control and subsequent aliquots (Figure 5). As Figure 6 shows, the magnitude of the effect differed between compounds and resulted in significant differences in resolution of impurities 10 and 3 eluted by the mobile phase that contained the trifluoroacetic acid solution from the first aliquot (R_s = 3.1) and the mobile phase that contained the unfiltered trifluoroacetic acid solution (R_s = 1.4).

Using the relationship between retention times and trifluoroacetic acid concentration shown in Figure 4 as a calibration curve, we used the change in retention time of the main peak and impurity 10 shown in Figure 5 to estimate the change in trifluoroacetic acid concentration in the extracted solution. The drop in retention of the main peak and impurity 10 was consistent with a 20% and 10% drop in trifluoroacetic acid concentration for the first and second 1-L aliquots, respectively. The decrease in retention times for impurities 10 and 3 in the first 2 L of extracted solution shown in Figure 5 was

fully consistent with decreased levels of trifluoroacetic acid in the extracted mobile phase (Figure 4). The titration of the mobile phase with sodium hydroxide also showed reduced levels of trifluoroacetic acid by 20% and 10% in the first and second 1-L aliquots, respectively.

Figure 7 shows the estimated changes in trifluoroacetic acid content from the extraction process based upon titration data and changes in HPLC retention. Because the retention behavior and the acid content of the later aliquots agreed with those of the unfiltered control, we concluded that adsorption, rather than evaporation, was responsible for a loss of trifluoroacetic acid during the extraction process. These data also indicate that as much as 2 L of dilute trifluoroacetic acid might be necessary to equilibrate the extraction disk before use for systems that are sensitive to trifluoroacetic acid concentration.

To further confirm that the adsorption of trifluoroacetic acid might be observed, we equilibrated the extraction disks by shaking them in a glass bottle that contained 800 mL of 0.044% trifluoroacetic acid in water overnight on an orbital shaker. The extraction disks were hydrophobic, so there was not the sort of intimate contact that occurs when the entire volume of liquid is pulled through the disk. Nevertheless, the mobile phase prepared from these solutions showed a small but significant decrease in the retention of the main peak compared with an untreated control, which is consistent with passive adsorption of trifluoroacetic acid by the PS–DVB extraction disk.

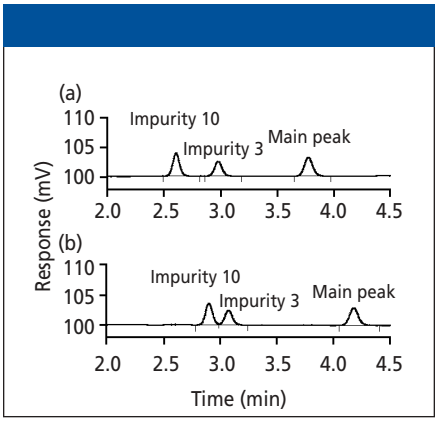

Figure 6: Comparison of chromatograms for method A eluted using (a) mobile phase extracted with a PS–DVB SPE disk and (b) unextracted, unfiltered mobile phase. The concentration of the main peak in this test mix was significantly reduced to assist in viewing low-level impurities.

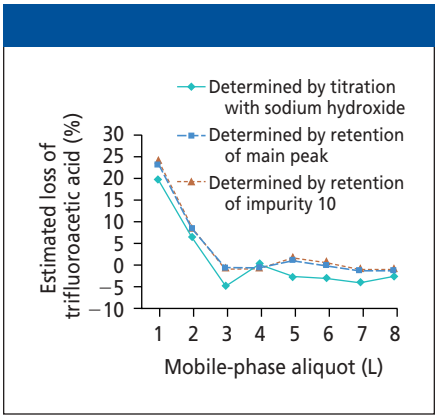

Figure 7: Change in trifluoroacetic acid concentration as a result of the extraction process, estimated by the change in solute retention (see Figure 4) and titration of aqueous mobile phase aliquots with 0.1 N sodium hydroxide, as compared with unextracted control.

Similar studies could assess whether this effect occurs with other organic mobile-phase additives, such as ion-pairing reagents, that typically are used in small amounts.

Conclusions

SPE is an effective procedure for removing common laboratory contaminants from mobile-phase components. When using PS–DVB disks, more than 95% of the background peaks are removed from blank chromatograms. Although this investigation evaluated the cleanup of extremely contaminated solutions, it is reasonable to assume that the extraction efficiency will be much greater in typical laboratory solutions with lower contaminant loads. Although we did not explicitly assess the extraction capacity of these disks, it is reasonable to extrapolate that the capacity is greater than 2 L for typical, less-contaminated solutions.

For most cases in this study, the use of the mobile-phase cleanup procedure had no effect on solute elution when compared with a glass-fiber-filtered control. Although the adsorption of trifluoroacetic acid could occur using this mobile-phase cleanup procedure, this effect can be overcome by pre-equilibration of the extraction disk as needed. Early incorporation of mobile-phase cleanup in the method development process not only maximizes the practical benefits but can help mitigate potential effects to system suitability caused by the procedure. A small adjustment of parameters — such as using extra trifluoroacetic acid in mobile phase A — early in method development could be all that is necessary to offset any changes in mobile-phase composition caused by the procedure.

The procedure is fast, inexpensive, and applicable to a variety of methods. Accordingly, this procedure has been implemented successfully in several laboratories at our company as a part of routine mobile-phase preparation. In many cases, chromatographic methods that are quite difficult to perform without the procedure can generate high-quality data on a routine basis with the use of this cleanup procedure.

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